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CONTRACTING ORGANIZATION: PowderJect Vaccines, Inc.
Madison, WI 53711

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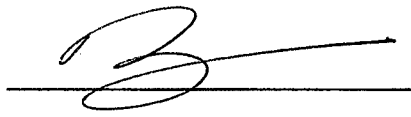

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13. ABSTRACT *(Maximum 200 words)*

Efforts in this funding year focused on gene gun-based induction of mucosal immune responses and enhancement of CTL.

Gene gun-based DNA immunization of rhesus macaques resulted in further refinement of DNA delivery parameters based on the degree of local inflammation. A comparison between mucosal and skin immunization revealed qualitative differences in localized mucosal responses but not peripheral blood responses. Efforts to enhance CTL responses resulted in the development of a gene gun-based method that induces high-frequency T cell responses in these animals. Studies were also initiated to evaluate the potential of a DNA immunization regimen as an adjuvant to highly active anti-retroviral therapy (HAART).

Gene gun-based DNA immunization of mice demonstrated enhancement of antigen specific CTL responses by co-delivering DNA encoding IL-6. Direct delivery of DNA to the tongue of mice demonstrated the tongue was not an inductive site for mucosal responses.

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TABLE OF CONTENTS

INTRODUCTION

Vaccination with DNA leads to *in vivo* expression of the encoded antigens (1-3) with the subsequent induction of specific humoral and cellular immune responses (4-7). The endogenous production of antigen following DNA delivery into the host cell mimics certain aspects of live attenuated vaccines without the safety and stability concerns associated with administration of an infectious agent.

Plasmid DNA can be introduced into a host by intramuscular (1, 3, 5, 8) or intradermal (42) inoculation. A more efficient method to introduce DNA is by direct intracellular delivery of plasmid DNA-coated gold beads into the skin (4, 6, 7, 9, 10) or mucosal surfaces (11, 12) using a gene gun. Skin and mucosa are ideal target sites for DNA vaccination. Both are populated by antigen presenting cells and immunologically competent for the elicitation of humoral and cytotoxic cellular immune responses (13, 14). Furthermore, the gene gun is highly efficient at inducing both types of responses following delivery of DNA to epidermal cells of the skin (2, 7, 9, 10).

The mucosal surface is a primary route for HIV infection. The largest mucosal surface in the body, the gut-associated lymphoid tissue (GALT), consists of organized lymphoid tissue as well as large numbers of activated CD4+ T cells and contains most of the lymphoid tissue in the body (15). Recent evidence has shown that the gastrointestinal tract is a major target site for SIV replication and early CD4+ T cell loss following intravenous inoculation with pathogenic SIV (16). These observations suggest that the gut-associated lymphoid tissue (GALT) is a crucial target site for early replication of immunodeficiency viruses following either intravenous or mucosal infection. A prophylactic vaccine against HIV is likely to be most effective if it induces an appropriate immune response at the site involved in the earliest stages of viral infection. Therefore, a candidate vaccine against HIV may need to induce mucosal immune responses.

The relationship between prognosis in HIV-1 infection and plasma viremia has been well-established (17). Although no single measure of immunity has been found to be predictive of *in vivo* control of HIV replication, evidence is mounting that cytotoxic T lymphocytes (CTL) play an important role. HIV infected patients with strong CTL responses demonstrate reduced acute viremia (18), and high levels of virus-specific CTL are associated with long term survival (19). In contrast, patients with low or undetectable CTL poorly control acute viremia and progress more rapidly to AIDS (20). These results strongly suggest that an efficacious prophylactic vaccine against HIV will need to induce cytotoxic T lymphocyte (CTL) responses.

A promising application of an HIV vaccine that induces CTL is in the setting of anti-viral therapy. Drug therapy of HIV-infected individuals can significantly reduce virus load with subsequent induction of T cell recovery and delay in disease (21). An immunization regimen that induces HIV-specific CTL may be effective during the T cell recovery associated with highly active anti-retroviral therapy (HAART). In particular, vaccine induction of CTL could effectively target residual infected cells and reduce or eliminate virus load.

Based on these observations, we believe induction of CTL and mucosal immune responses offer the most promising strategies for the development of an efficacious vaccine against HIV. Therefore, during the period of September 30, 1997 to September 29, 1998, our research efforts have focused primarily on methods to enhance CTL responses and induce mucosal immune responses by gene gun-based DNA immunization. In addition, we initiated 2 challenge studies in the SIV macaque model to test the role of CTL and mucosal immune responses in protection. These efforts emerged in pursuit of the specific aims suggested in our contract

RESULTS

Nonhuman Primate Model:

Background:

Most of our efforts have focused on the simian immunodeficiency virus (SIV) macaque model for AIDS because it allows investigation of vaccine protection against both infection and disease. We previously demonstrated that rhesus macaques immunized with a vaccine regimen consisting of multiple DNA inoculations exhibited suppressed viral load, attenuated CD4+ T cell decline, and delay in progression to disease following challenge with a heterologous, pathogenic SIV (22). In our third funding year (96-97), we reported that this regimen induced suppressed antibody responses in the presence of enhanced CTL. These results suggested that CTL may have played an important protective role in these animals.

Having identified a DNA immunization regimen that induced CTL responses, we sought to evaluate this regimen as an adjuvant to HAART in SIV-infected monkeys. The acyclic nucleoside phosphonate analog, PMPA, reduces virus load in the SIV-infected rhesus macaques and closely approximates the antiviral state achieved by current drug therapies in HIV-infected humans (23, 24). During this funding year, we initiated studies in this model to test the potential of DNA vaccines to induce CTL and prevent or reduce re-emergence of viremia and subsequent disease after termination of drug therapy. Initial results demonstrate that prior to infection and treatment with PMPA, multiple DNA inoculations induced CTL in 15 of 20 monkeys, with variation in CTL antigen specificity between monkeys.

During the previous funding year, we initiated studies to evaluate the potential of gene gun-based DNA immunization to elicit localized mucosal immune responses by specifically targeting mucosal tissue. Recent reports indicate that gene gun DNA inoculation to the skin may also be capable of inducing mucosal immune responses (Olsen). In addition, delivery of DNA to mucosal surfaces appears to elicit both mucosal and peripheral immune responses (11, 12). Based on these observations, we sought to determine if mucosal inoculation provided an advantage over skin immunization. During this funding year, we merged this mucosal study with a parallel group of skin-immunized monkeys to compare skin versus mucosal DNA inoculation for the elicitation of peripheral (i.e. responses localized in the peripheral blood) or mucosal immune responses (responses localized in the lamina propia). Analysis of CTL responses demonstrated that while mucosal inoculation is a superior site to skin for the induction of localized gut CTL, both skin and mucosal inoculation are equally effective for the induction of peripheral blood CTL.

In this funding year, we initiated studies to further improve the induction of CTL in rhesus macaques by gene gun-based DNA immunization. These studies further optimized gene gun delivery parameters to monkeys based on the local inflammatory response (erythemia) and developed a method for inducing high frequency primary CTL responses. This method, which combines a novel epitope expression vector with a

recombinant viral vector (modified vaccinia Ankara or MVA) in a prime-boost regimen, resulted in maximum specific CTL frequencies of 10-18% of total CD8+ T cells. These levels exceed that induced by acute SIV infection (27) and to our knowledge, are the highest levels of vaccine-induced CTL responses ever detected in a large animal model.

Recent Data:

DNA immunization as an adjuvant to HAART in SIV-infected rhesus macaques: CTL responses are crucial for elimination of virus once infection occurs. Therefore, an immunization strategy that aims to eliminate residual virally infected cells in the setting of HAART will likely need to induce potent CTL responses at the time of T cell recovery. However, previous studies demonstrated that DNA immunization required multiple doses and a minimum of 24 weeks to induce maximum CTL responses in rhesus macaques (22, 28, 29). Because of this time requirement, we chose to immunize monkeys with a sufficient number of DNA doses to induce CTL prior to SIV infection and the relatively short treatment (4 weeks) with PMPA.

In this study, 20 monkeys received 9 consecutive doses of DNA spaced 8 to 12 weeks apart. Each dose of DNA consisted of an expression vector encoding the macrophagetropic sequences from $SIV_{17E\text{-}FR}$ *gag-pol-env*. Three weeks following the final inoculation, effector mononuclear cells were purified from peripheral blood by Ficoll-Paque barrier density centrifugation for analysis of CTL responses against EBV-transformed autologous B cell targets infected with a recombinant vaccinia expressing SIV *gag*, *pol*, or *env*. Preliminary data in Table 1 shows detection of CTL for each monkey. Animals were scored as positive for CTL when % specific lysis values were greater than 10 percentage points over background lysis against autologous B cell targets infected with a control vaccinia. Consistent with earlier findings, CTL were detected in most animals following a multiple dose immunization regimen. After 8 doses, we detected CTL in 15 of 20 monkeys (80%). Levels of CTL were similar for all monkeys. However, the specificity of the CTL response varied between monkeys, with most responding to either *gag* (7 monkeys) or *env* (9 monkeys).

These monkeys were divided into two study groups: DNA ONLY and DNA + PMPA. Over the next funding period, animals in both groups will be bled again 6 weeks following the booster immunization for CTL analysis and then challenged intravenously with SIV. The DNA + PMPA group will begin daily treatments with PMPA starting one day after the challenge. The DNA ONLY group will be left untreated. At the time of challenge ($t = 0$), we will add 14 naïve monkeys to this study. Ten of these animals will be challenged with SIV and receive PMPA treatment starting one day after challenge (PMPA ONLY) and the remaining 4 naïve animals will provide infected, untreated controls. The efficacy of the DNA-based immunization strategy in the context of HAART will be evaluated in terms of induction of relative levels of antigen-specific CTL before and at various time points after challenge, and in terms of virus load by quantitative-competitive PCR and by clinical measures of disease progression.

Study Group	*Monkey*	*CTL*	*Antigen specificity*
DNA ONLY	**7197**	+	**Gag**
	7997	+	**Gag**
	7497	+	**Env**
	7897	+	**env, pol**
	8397	+	**env, gag**
	9097	-	
	7397	-	
	8197	+	**env, gag**
	8597	+	**Env**
	8797	+	**Gag**
	8997	+	**Pol**
DNA + PMPA	**6997**	-	
	7597	+	**Env**
	6597	+	**Gag**
	6497	-	
	6697	+	**gag, pol, env**
	6797	+	**Env**
	6897	+	**Env**
	8297	+	**Env**
	8897	-	

Table 1: CTL induction and specificity in monkeys immunized with DNA expressing $SIV_{17E\text{-}FR}$ *gag-pol-env*

Comparison of skin versus mucosal immunization for induction of CTL, mucosal immune responses, and protection in rhesus macaques:

Efforts in the SIV/macaque model have demonstrated that transient infections resulting from mucosal exposure to limiting doses of infectious SIV can protect rhesus macaques from subsequent intrarectal challenge with pathogenic levels of SIV (30). Furthermore, similar studies have provided evidence of an association between mucosal protection and the induction of gut-associated CTL in the lamina propia (31). In addition to skin, the gene gun can also access and deliver DNA to the cells of the mucosal tissue by directly targeting its surface. In our last funding year (96-97), we reported the induction of CTL responses in the lamina propia in 3 of 4 monkeys following DNA inoculation into mucosal tissue. These results demonstrated that direct targeting of these tissues with the gene gun could induce a specific mucosal response. During this funding year, we continued evaluation of the induction of mucosal immune responses by gene gun DNA immunization.

Based on recent data suggesting gene gun inoculation to the skin may be capable of inducing mucosal immunity (25), we sought to determine if targeting mucosal tissue offered an advantage over targeting skin. Toward this end, we merged these mucosal-immunized monkeys with a second group of three monkeys that had been receiving parallel skin immunizations with the same SIV-FR construct. All monkeys had received 4 consecutive DNA immunizations spaced 12-14 weeks apart. To compare CTL responses

in the lamina propia and peripheral blood, skin-immunized monkeys were boosted with a 5th dose consisting of 10.0 μg of SIV-FR DNA delivered to the abdominal epidermis. At the same time, mucosal-immunized monkeys also received a 5th dose consisting of 16.0 μg SIV-FR delivered to the inner cheek (6.0 μg), tongue (6.0 μg) and rectum (4.0 μg). Four weeks following the boost, a 2 cm section of jejunum and accompanying mesenteric lymph node were surgically removed from 5 of these animals. Due to limitations in the number of gut resection surgeries that can be performed on a single monkey, 2 mucosal-immunized monkeys (L999, L978) did not undergo additional surgery following the 5th dose. Mononuclear cells were purified from the intestinal and lymph node tissue and evaluated for CTL activity against EBV-transformed autologous B cell targets infected with a recombinant vaccinia expressing SIV gag or env. The results are shown in Figure 1 below.

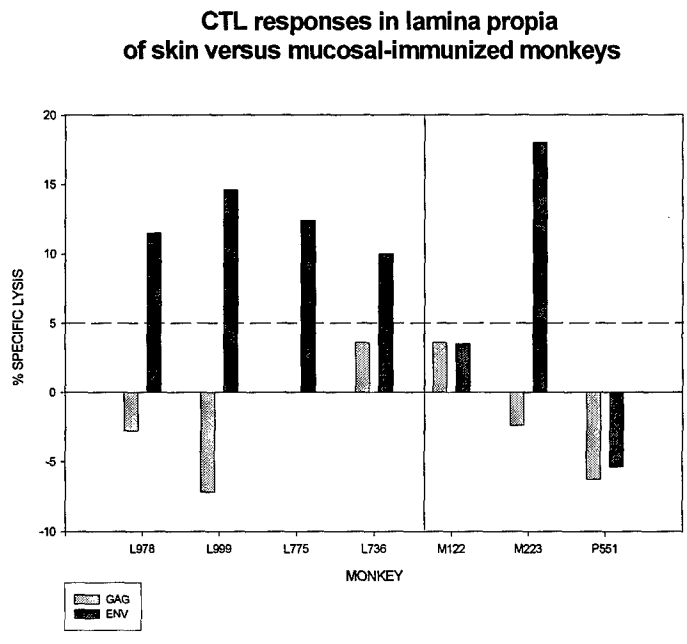

Figure 1: SIV-specific CTL activity in the lamina propia from rhesus macaques following 4 (L999, L978) or 5 (L736, L775, M122, M223, P551) consecutive gene gun DNA immunizations in the mucosa or skin with the expression vector SIV-FR (*gag-pol-env*). CTL responses are reported in % specific lysis at an effector to target ratio of 20:1. Levels above 5% (------) are considered positive in this assay.

We detected gut-associated CTL responses in 4 of 4 monkeys immunized in mucosal tissue and 1 of 3 monkeys immunized in the skin. These results demonstrate that although DNA inoculation to the skin can induce specific mucosal responses distal to the site of delivery, targeting DNA directly to the mucosal tissue is more efficient in the induction local mucosal immune responses.

To compare the efficacy of skin versus mucosal immunization for the induction of peripheral blood CTL, we increased the number of skin-immunized monkeys from three to seven to improve statistical significance. The four monkeys added to the study had received parallel immunizations consisting of identical plasmid DNA, dose, and intervals between inoculations. We immunized each monkey in the skin or mucosa with 3 additional inoculations of DNA (for a total of 8) to maximize the CTL response. Four weeks following the final immunization, peripheral blood mononuclear cells were isolated from each monkey and tested for CTL activity against autologous B cells infected with recombinant vaccinias as described above. The results are shown in figure 2 below:

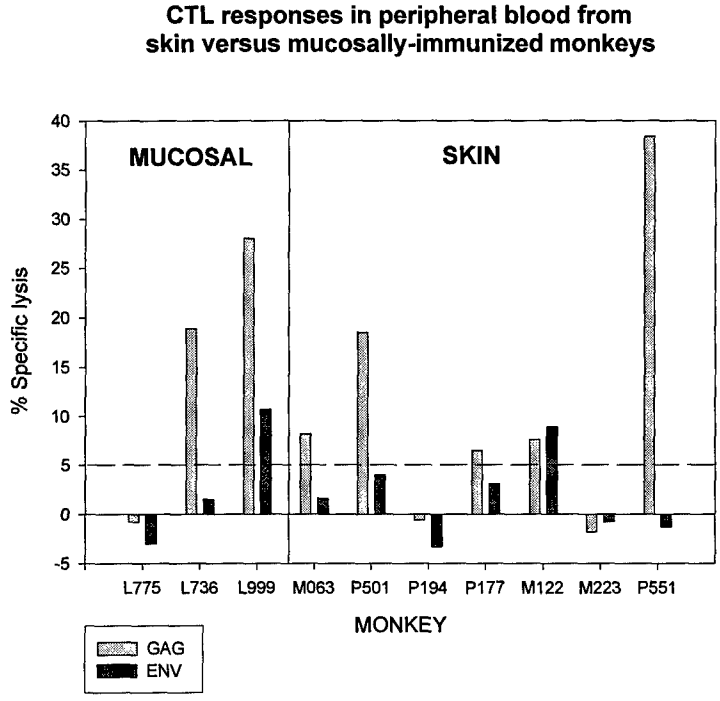

Figure 2: SIV-specific CTL activity in the peripheral blood from rhesus macaques following 8 consecutive gene gun DNA immunizations in the mucosa or skin with the expression vector SIV-FR (*gag-pol-env*). CTL responses are reported in % specific lysis at an effector to target ratio of 20:1. Levels above 5% (------) are considered positive in this assay.

We detected specific peripheral blood CTL in 2 of 3 mucosally-immunized monkeys and 5 of 7 skin-immunized monkeys. CTL levels were similar between the two groups and greater than the levels seen in gut-associated tissue. The stronger CTL detected in the peripheral blood may be due to more efficient isolation of mononuclear cells from the peripheral blood than from the lamina propia. These results suggest that inasmuch as direct inoculation of DNA to the mucosa is more efficient than skin inoculation for the induction of localized mucosal immune responses, it is equally as efficient as skin

inoculation for the induction of peripheral responses. Interestingly, although the expression plasmid used expressed both *gag* and *env*, CTL detected in the lamina propia were specific for only *env*. In contrast, CTL detected in the peripheral blood was predominantly specific for *gag*. This result may be due to differences in antigen processing and presentation between cells of the mucosal associated lymphoid tissue (MALT) and those of the skin (SALT).

We recently challenged these animals with pathogenic SIV via intrarectal inoculation to compare these immunization strategies for the induction of protection against infection or disease. Initial data are not yet available. However, CTL and mucosal responses varied in this study between animals from strong to undetectable. Therefore, this challenge may provide valuable insight into the role of CTL or mucosal immune responses for protection against immunodeficiency viruses.

Optimization of gene gun delivery parameters based on localized inflammation at the target site: Our results consistently show that multiple DNA doses are required to elicit potent CTL responses in nonhuman primates, demonstrating a need to identify strategies to improve the induction of CTL in monkeys. To overcome this obstacle, we sought to enhance overall induction of immune responses in nonhuman primates by further refinement of gene gun delivery parameters. Gene gun administration of DNA to the skin results in a mild, localized inflammatory response as indicated by the induction of transient erythema at the target skin site. In the previous funding year, we reported an association between the local inflammatory response, or degree of erythema, and the induction of immune responses in pigs. Since we have also observed a similar association in nonhuman primates, we attempted to determine the optimum degree of erythema for efficient induction of immune responses in rhesus macaques.

Toward this aim, we designed an experiment that combined several gene gun delivery parameters to induce a broad range of degrees of erythema at the target skin site. Twelve monkeys were immunized using a combination of parameters that had been shown to have consistent influences on the level of erythema. Parameters in this study that increased versus decreased erythema at the skin target site are listed below in Table 2.

INCREASE ERYTHEMA		*DECREASE ERYTHEMA*	
Parameter	**Experimental Application**	**Parameter**	**Experimental Application**
Increase number of gold particles per target	1.0 mg gold / target	Decrease number of gold particles per target	0.25 mg gold / target
Increase delivery pressure	500 psi	Decrease delivery pressure	300 psi
Target site with thin, softer skin	Skin over inguinal lymph node (LN)	Target site with thicker, tougher skin	Abdominal skin (A)

Table 2: Parameters affecting the intensity of erythema at the target skin site within 30 seconds following gene gun delivery of DNA-coated gold beads. Parameters that increase or decrease erythema increase or decrease the overall intensity of redness at the target site, respectively. The experimental applications of these parameters are the specific methods used in this study to increase or decrease the relative intensity of erythema. Various combinations of these methods were used to manipulate the overall degree of erythema induced in each animal. The degree to which each parameter affects erythema is not necessarily equal.

As shown in Table 3, we deliberately manipulated and combined parameters in each monkey to induce broad range of degrees of erythema among the animals. Degrees of erythema were scored 30 seconds after each inoculation and a final erythema score was determined by averaging the scores from multiple inoculations (including prime and boost) in the same animal (Table 3).

Monkey	*Mg gold per target*	*psi*	*Site*	*Erythema score**	*mIU / ml sAg*
N031	**0.25**	**300**	**A**	**0.5**	**1.5**
P166	**0.25**	**300**	**A**	**2.5**	**2960**
L937	**0.25**	**300**	**LN + A**	**2**	**5**
M212	**0.25**	**300**	**LN + A**	**0.5**	**10**
N112	**0.25**	**300**	**LN**	**3**	**96**
N706	**0.25**	**300**	**LN**	**3**	**214**
N056	**1.0**	**500**	**A**	**2**	**91**
L695	**1.0**	**500**	**A**	**1.5**	**3.4**
L785	**1.0**	**500**	**LN+A**	**2**	**148**
M278	**1.0**	**500**	**LN+A**	**2.5**	**476**
M192	**1.0**	**500**	**LN**	**3**	**3000**
L842	**1.0**	**500**	**LN**	**4**	**406**

Table 3: Influence of parameter combinations on erythema score. The degree of erythema was qualitatively scored 30 seconds following delivery on a relative redness scale of 1 to 4 where 1 = approximately 25% of the target site is red, 2 = 50%, 3 = 75%, and 4 = 100%. All targets were delivered into the skin sites over the lymph node (LN), on the abdomen (A), or a combination of both (LN + A). *Erythema score is a compiled average of all target sites following each of 2 immunizations spaced 12 weeks apart.

We chose to immunize these monkeys with two doses (prime and boost) of DNA expressing hepatitis surface antigen (HBsAg) because immunization with HBsAg DNA results in the induction of antibody responses in monkeys after only one or two doses. This rapid seroconversion is therefore an advantage for efficient optimization of delivery parameters in monkeys. Two weeks following the final booster immunization, plasma was collected from each monkey and antibody responses to HBsAg determined using an AUSAB kit (Abbott). Antibody responses to HBsAg shown in Table 3 were plotted against erythema score. The results are shown in Figure 3 below:

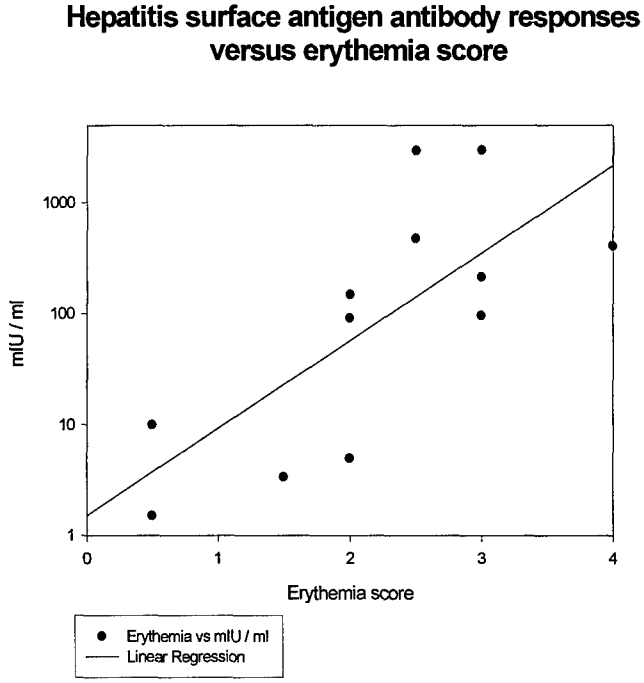

Figure 3: Linear regression analysis of hepatitis antibody response versus erythema score for each monkey.

These results demonstrate that increasing the local inflammatory response at the skin target site has a beneficial impact on the induction of immune responses following gene gun delivery. Since local inflammation results in the recruitment of antigen presenting cells to the affected site, we propose that the transient erythema observed following gene gun delivery increases antigen processing and presentation at the target site. These results suggest that the gene gun-mediated induction of immune responses might be further enhanced by manipulation of dendritic cells *in vivo*, including co-delivery of chemical adjuvants or chemokines to promote recruitment of these antigen presenting cells (APC). In addition, since gene gun DNA immunization likely results in the direct transfection of epidermal dendritic cells (32), alternative strategies may include pre-treatment of the target site with various methods that will increase gene gun targeting of APC.

Development of a method for that induces high frequency primary CTL responses in nonhuman primates. The requirement for multiple DNA inoculations to induce CTL in rhesus macaques responses in our studies may be partly due to the observation that there appears to be a unique reciprocity between HIV and SIV-specific CTL and antibody responses following gene gun-based DNA immunization (6, Annual Report 1996-97). We hypothesize that antibodies arising early in the immunization regimen suppress CTL. However, following multiple inoculations, modest CTL are boosted sufficiently to mediate the reciprocal suppression of antibody. To overcome this reciprocity and induce CTL earlier in the immunization regimen, we sought to enhance the induction of CTL by evaluating the potential of immunizing with expression vectors encoding specific CTL epitopes.

To induce CTL, antigen presenting cells process virus-derived antigens by proteolysis into 8- to 10- amino acid residues-long peptides that are presented in context of major MHC class I molecules. The fact that only small parts of antigens are sufficient to induce CTL has been exploited in the construction of experimental polyepitope-based vaccines (33, 34, 35). Unlike the use of whole antigens, this approach allows focus of the cytotoxic cellular immune responses toward highly conserved and important epitopes and excludes peptide domains that could induce suppressive antibody responses.

One disadvantage associated with CTL epitope-based vaccines is the lack of T helper cell epitopes that cooperate with cytotoxic T cells to increase the potency of the response against a given epitope. Indeed, peptide antigens often require conjugation to an immunogenic carrier moiety to elicit efficient immunogenicity. Several studies have demonstrated that the hepatitis B virus nucleocapsid antigen (HBcAg) may represent an efficient carrier moiety (36). Indeed, the HBcAg particle induces potent immune responses in humans and experimental animals (37) and studies in mice have demonstrated that the protein antigen HBcAg elicits strong T helper cell responses (38). This enhanced immunogenicity may be due to the ability of HBcAg to self-assemble into an immunogenic particle (39). For this reason, HBcAg has been used as a carrier moiety for translationally fused heterologous peptide B cell epitopes (39, 40). Based on these observations, we sought to evaluate the potential of enhancing DNA vaccine-induced CTL responses in rhesus macaques by inserting an SIV gag-specific, Mamu A*01-restricted CTL epitope (p11C: CTPYDINQM) (27) into an internal position of a carrier HBcAg expression vector (HBcAg-A*01).

To test for induction of CTL responses against the inserted epitope, 3 rhesus macaques were primed with an expression vector encoding a multi-epitope polypeptide consisting of mouse, human, and macaque CTL epitopes, including the p11C epitope described above. This construct, called pTH.HW, was generated by inserting the C...M epitope into the expression vector previously described as pTH (41). Two monkeys (95045 and 95058) received 4 booster immunizations consisting of a co-delivery of the pTH.HW vector with the hepcAg-A*01 vector. The third monkey (96031) received 2 identical booster immunizations. The first booster immunizations were administered 6 (96031) or 13 (95045, 95058) weeks after the prime. Subsequent booster immunizations were administered 4 to 6 weeks apart in all animals. Peripheral blood mononuclear cells were isolated and stimulated *in vitro* for 2 weeks with peptide-pulsed EBV-transformed autologous B cells. The percentage of C...M-specific CD8+ T cells was quantitated for each monkey by flow cytometry using FITC-coupled tetrameric Mamu A*01 / p11C, C-M complex as previously described (27). In addition, C...M-specific cytolytic activity was determined by measuring lysis against peptide-pulsed autologous B cells in a ^{51}Cr-release assay. The results are shown in Figure 4 below.

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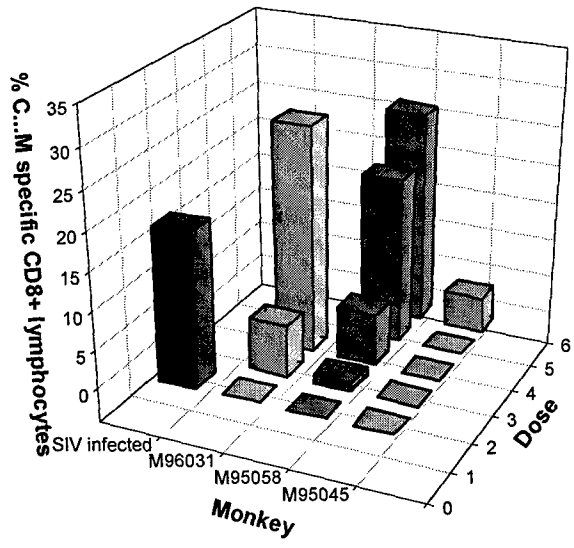
A

Tetramer staining of *in vitro* stimulated specific CD8+ T cells in DNA-immunized Mamu A*01 monkeys



In vitro restimulated CTL responses in DNA-immunized Mamu A*01 monkeys

B

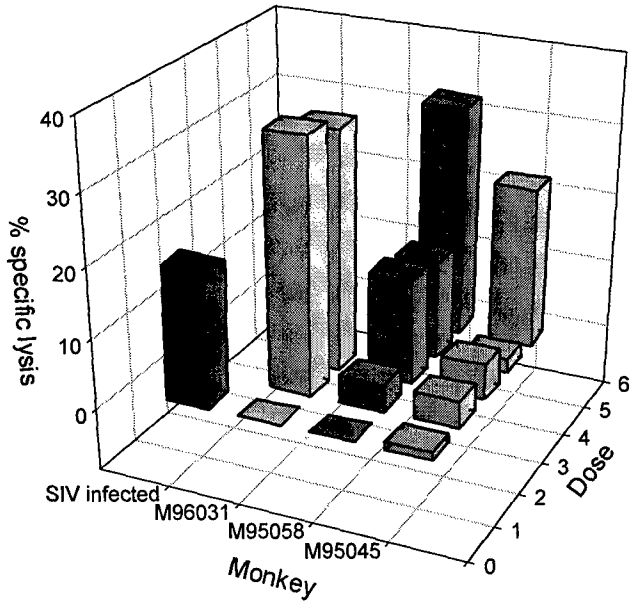


Figure 4: Induction of epitope-specific CD8+ T lymphocytes in an SIV infected monkey and three DNA-vaccinated monkeys. CD8+ T cell responses were measured after each of 5 DNA inoculations for monkeys 95058 and 95045 and each of 3 DNA inoculations for monkey 96031. PBMC were stimulated for 2 weeks *in vitro* and then stained with tetrameric complexes (Panel A) or tested in standard ^{51}Cr-release assays (Panel B).

These results show that a single inoculation with the pTH.HW vector resulted in detectable CTL responses. However, a second inoculation with a combination of the pTH.HW vector and the hepcAg-A*01 vector resulted in a significant increase in these responses. Additional DNA vaccinations with both vectors effectively boosted these

responses in two of these monkeys (95058, 96031) to levels greater than that detected in an SIV-infected monkey. These results suggest that a DNA-based immunization strategy is capable of inducing CTL responses equal to or greater than a viral infection. Furthermore, these results support an epitope-based strategy for the efficient induction of CTL by DNA immunization.

We hypothesized that the HBcAg functioned as an adjuvant to enhance responses to this epitope. To evaluate this potential role of HBcAg, we measured specific IgG responses generated against cAg in these animals. Figure 5 shows a direct correlation between the immunogenicity against the cAg of the carrier vector (cAg IgG) and the level of C...M-specific CTL in each animal and after each immunization. These results provide evidence for an adjuvant role of HBcAg in this strategy.

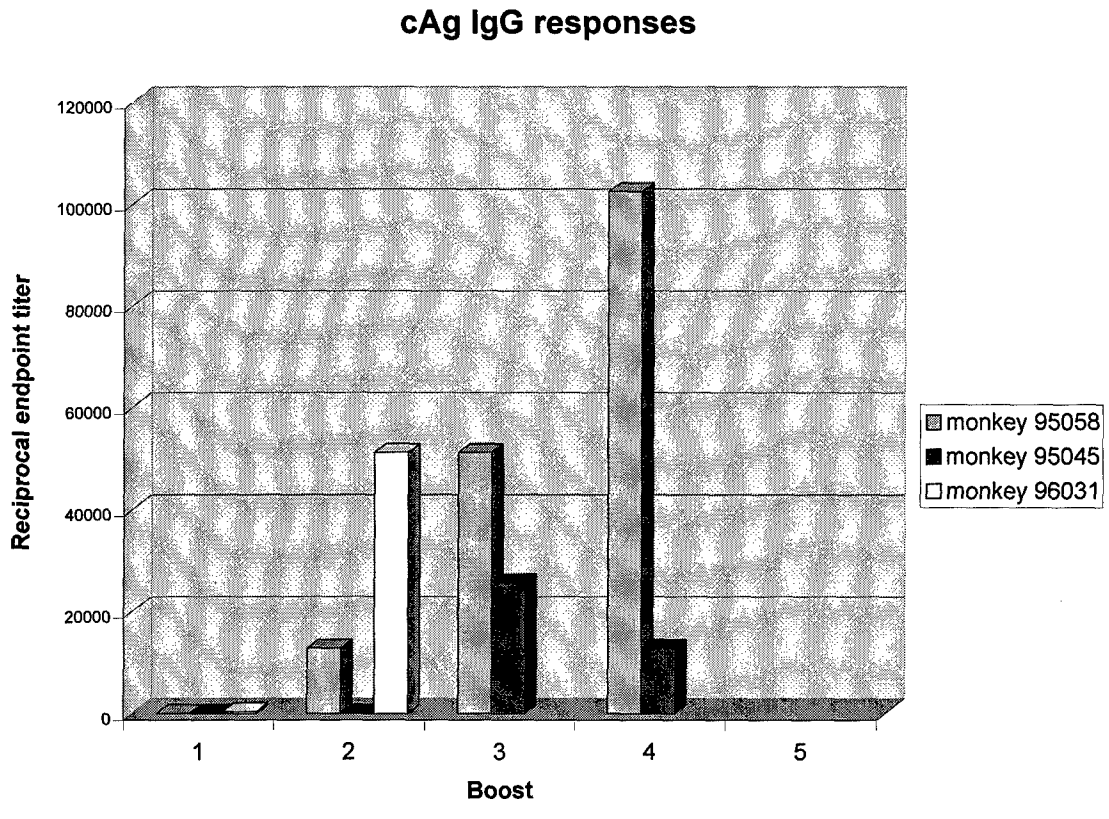


Figure 5: HBcAg-specific IgG responses in monkeys monkeys vaccinated with the HBcAg-A*01 carrier plasmid. Monkeys were primed with the pTH.HW expression vector. The second dose (boost 1) represents the primary immunization for HBcAg-A*01.

We have reported that combining DNA with recombinant viral vectors in a prime-boost regimen results in the induction of synergistic responses (22). Recently, Hanke et. al. reported enhancement of epitope-specific CTL responses following a similar DNA prime / MVA (modified vaccinia Ankara) boost vaccination regime (34). To determine if an

MVA booster immunization enhance CTL responses in these monkeys, we administered 5 X10^8 p.f.u. of a recombinant MVA encoding the HW polyepitope genes), including C...M (MVA-HW). Table 5 shows the induction of very high frequency tetramer staining (10 and 18 %) in fresh, unrestimulated PBMC in two of these monkeys. Furthermore, we were able to detect primary killing of peptide-pulsed autologous targets by unrestimulated PBMC isolated from these monkeys after the MVA boost.

Monkey	*Tetramer Staining* *(Fresh, unrestimulated PBMC)*	*Cytolytic Activity* *(Frozen, unrestimulated PBMC)*
95058	**18.0%**	**14%**
96031	**8.3%**	**7%**
95045	**0.8 %**	**- 2%**

Table 4: Tetramer staining and cytolytic activity from fresh or frozen unrestimulated. Monkeys were immunized with 5 (95058, 95045) or 3 (96031) DNA inoculations followed by 1 inoculation with recombinant MVA-HW. PBMC were assayed one week following MVA inoculation.

Based on these results, we have identified a gene gun-based immunization regimen that induces very high frequency CTL responses against viral epitopes. These levels exceed levels induced by acute SIV infection. Based on these frequencies and the detection of primary lysis from unrestimulated PBMC, we believe these responses may be the highest levels of vaccine-induced CTL ever detected in a nonhuman primate model.

Murine Model

Background: A number of reports have demonstrated that immune responses induced by DNA delivery can be manipulated by co-delivery of various cytokine expressing genes (43), the resting period between immunizations (44), the number of doses administered (6), and the route of DNA delivery (45). However, few of these studies have reported the effects of such manipulations on protection against infection.

We recently reported that co-administration of DNA encoding interleukin-6 and genes for influenza substantially improved protection against influenza infection following a mucosal (intranasal) challenge (25). We also reported that administration of DNA to the tongue of pigs was superior to skin DNA administration for protection against mucosally administered swine influenza (12). However, the mechanisms mediating these apparent improvements were not identified. IL-6 is important for end-stage differentiation of B cells and studies have demonstrated that IL-6 is critical for the induction of mucosal IgA responses (46). However, IL-6 also stimulates proliferation of T cells (47). Therefore IL-6 may enhance immunity to influenza by increasing both IgA for protection from infection at mucosal surfaces and cellular immune responses for viral clearance. The enhanced protection in pigs following delivery of DNA to the tongue suggests the tongue may be an inductive site for mucosal immunity.

To evaluate the immune effects of IL-6, we delivered DNA encoding hepatitis B surface antigen DNA encoding human IL-6 or control (empty) vector. In parallel we also evaluated the tongue as an inductive site for mucosal immune responses by including mice immunized with HBsAg DNA codelivered with IL-6 or control DNA.

Five groups of Balb/ C mice each received 2 gene gun DNA immunizations at weeks 0 and 4 as follows:

- Group 1: HBsAg + IL-6 delivered to the tongue
- Group 2: HBsAg + Control vector delivered to the tongue
- Group 3: HbsAg + IL-6 delivered to the skin
- Group 4: HbsAg + Contol vector delivered to the skin
- Group 5: Control mice: Control vector or IL-6 vector delivered to either tongue or skin.

We compared the induction of total IgG, IgG1, IgG2a, serum and mucosal IgA, T helper responses, and CTL in these animals. Antibody and T helper responses are summarized in Table 5.

	Skin (S) vs. Tongue (T)	*Effect of co-delivery with IL-6**
Antibody titer	**T = S**	**No effect**
IgG1	**S > T**	**Increase**
IgG2a	**T >/= S**	**Decrease**
IFN	**S > T**	**No effect (S), Increase (T)**
IL-4	**Little or non detected**	**Increase (S), No effect (T)**
IgA (serum and secretory)	**Low or none detected for all specimens**	**No effect**

Table 5: Evaluation of HbsAG-specific responses following gene gun delivery of DNA to the tongue or skin and the co-delivery of IL-6. > (relative increase in response), < (relative decrease in response), = (equivalent responses). Antibody titers, IgG1, IgG2a, serum IgA were measured by ELISA against recombinant HBsAg. Secretory IgA responses were measured by ELISA in the vaginal washes and saliva collected 1 or 2 weeks post-immunization. IFN and IL-4 release were measure by ELISA of supernatant of *in vitro* rHBsAg stimulated splenocytes.

Antibody responses were not affected by skin or tongue immunization or by co-delivery with IL-6. In addition, there was no clear influence in any of these groups on the T helper cells responses, as indicated by IFN and IL-4 and by antibody isotype (IgG1, IgG2a). A significant finding was that neither the tongue nor IL-6 co-delivery resulted in the induction of detectable secretory IgA responses. These results indicate the enhanced protection seen with IL-6 and tongue delivery is not due to induction of localized mucosal immunity. These results further suggest that the tongue is not an inductive site for mucosal immunity. However, these results do not account for potential differences between the mouse and pig model. Additional pig studies will be required to elucidate the mechanisms for enhancement of protective immunity by tongue inoculation.

Although co-delivery of IL-6 had no effect on the T helper or antibody responses in these animals, it had a potent positive effect on the induction of CTL (Table 6). These results suggest that the enhanced protection against influenza associated with co-delivery of IL-6 DNA may be mediated by CTL. Future studies will utilize IL-6 to enhance CTL in the SIV model for AIDS. In addition, additional studies will aim to determine if there is a correlation between IL-6-mediated enhancement of protection and enhancement of specific CTL. Over the next year, murine studies will continue to provide valuable information on methods to enhance specific immune responses for application in the development of a candidate vaccine against HIV.

CONCLUSION:

To effectively test candidate DNA vaccines in the SIV model for AIDS, we focused our efforts toward specific aim 1 (optimization of DNA immunization parameters in animal models) on further refining parameters for eliciting maximum responses in the rhesus macaque. Work toward this aim evaluated the role of local inflammation at the target site (erythemia) for the induction of immune responses and optimized the level of erythemia needed to maximize these responses.

Work towards specific aim 2, elicitation of protective immune responses in the pig as a model for DNA immunization of human skin, was completed during our last two report periods. We documented these data in reports submitted in September of 1996 and October of 1997. In summary, gene gun-based DNA immunization induced protection in pigs against swine influenza (12) and induced protective levels of neutralizing antibody against hepatitis B (9).

We continued to use the nonhuman primate model for work towards specific aim 3, the development of DNA-based vaccines for AIDS by induction of HIV-1 and SIV-specific immune responses in rhesus macaques. Work toward this aim resulted in development of a gene gun-based method that induces very high frequency CTL responses against specific SIV epitopes in monkeys. In addition, we continued to evaluate the potential to elicit mucosal, peripheral, and protective immune responses by comparing direct DNA delivery to mucosal versus skin surfaces in these animals. We also initiated studies to evaluate the potential of a DNA immunization regimen that induces CTL to act as an adjuvant in the setting of highly active anti-retroviral therapy (HAART). Finally, we initiated three challenge studies to test the efficacy of these strategies for protection against SIV.

The murine model was employed toward specific aim 4, the modification or augmentation of humoral or cytotoxic cellular immune responses by co-delivering cytokine encoding genes with antigen expressing vectors. Work toward this aim focused on strategies to enhance CTL responses by co-delivery of DNA expressing the cytokine IL-6. In addition, we used the murine model to evaluate the tongue as an inductive site for mucosal immune responses.

During the final year of our grant, we will obtain valuable challenge data in the nonhuman primate model to evaluate the potential for CTL to protect against infection or disease, or control virus in the setting of highly active antiretroviral therapy. A

particularly promising strategy that emerged during this period was the development of a novel method to induce high frequency CTL responses in monkeys. Over the next year, we plan to continue to explore this method in the context of development of a polyepitop-based DNA vaccine. In addition, we plan to continue to employ the murine model to evaluate methods for enhancement of specific immune responses including the development of novel expression vectors and evaluation of the potential to co-deliver classical adjuvants or chemokines.

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PRINCIPAL INVESTIGATOR: Dexiang Chen, Ph.D.
ANNUAL REPORT DATE: October 29, 1998

ADDENDUM: Table 6

Target site	*HBsAg + IL-6 (% specific lysis - control lysis)*	*HBsAg + Control (empty) vector (% specific lysis - control lysis)*
Skin	**49.8**	**19.7**
Skin	**48.5**	**32.5**
Skin	**27.2**	**34.0**
Tongue	**24.9**	**-7.7**
Tongue	**30.5**	**14.0**
Tongue	**19.2**	**1.2**

Table 6: Percent specific lysis against peptide-pulsed target cells at an effector : target ratio of 50/1 was compared in mice immunized with a codelivery of HBsAg + IL-6 DNA vs. mice immunized with a codelivery of HBsAg + control (empty) vector DNA. Control lysis against unpulsed targets was subtracted. Mice were immunized in the skin or tongue as indicated. A statistical difference between these groups was determined by paired T-test analysis ($p < 0.05$)

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REPLY TO
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1 JUN 2001

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FOR THE COMMANDER:

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PHYLLIS M. RINEHART
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